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Laboratoire Vancouver Laboratory
6620 N. W. Marine Drive
Vancouver, B.C. V6T 1X2
Telephone (604) 224-3221
Telex 04-508552

Laboratoire Ottawa Laboratory
800 Montreal Road
Ottawa, Ontario K1G 3Z5
Telephone (613) 744-0963
Telex 053-3606

Forintek Canada Corp.

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UTILIZATION OF HEMICELLULOSE FROM WOOD AFTER ORGANOSOLV TREATMENTS

November, 1986

J.N. Saddler
Project Leader

J.N. Saddler
Department Manager

UTILIZATION OF HEMICELLULOSE FROM WOOD AFTER ORGANOSOLV TREATMENTS

RENEWABLE ENERGY DIVISION

PREPARED FOR:

Bioenergy Development Program
Renewable Energy Division
Coal and Alternative Energy Branch
Energy, Mines and Resources Canada
Ottawa, Canada
DSS Contract File No.: 05SR.31926-4-5025
(December, 1986)

PREPARED BY:

Maria K.-H. Chan, J. Jeffrey Wilson,
and Ernest K. C. Yu

Biotechnology Department
Eastern Laboratory
Forintek Canada Corporation
800 Montreal Road
Ottawa, Ontario
K1G 3Z5
(613)744-0963

SCIENTIFIC AUTHORITY:

Ralph P. Overend
Division of Biological Sciences
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PREFACE

This report details the results of a two year study by Forintek Canada Corporation of the chemical characterization and potential fermentability of the spent liquors produced as a waste stream from the organosolv pulping process. Fermentation of the hemicellulose sugars present in these spent liquors to produce a marketable liquid fuel product such as ethanol or butanediol could make the organosolv process more economically attractive as an alternative pulping process. A series of spent liquor samples (15) were prepared by Econotech Services Limited, New Westminster, British Colombia, for analysis of carbohydrate and lignin content as well as fermentability by the bacterium Klebsiella pneumoniae to produce butanediol and ethanol.

The results confirmed the inhibitory nature of organosolv spent liquor when used as a fermentation substrate. Similar inhibition has also been reported by Econotech Services Limited in their fermentation studies which employed different microorganisms. The unknown compounds in the spent liquors which are toxic to the fermentative microorganisms were found to be closely associated with sugar degradation materials produced during the organosolv process and not the lignin degradation products, as originally proposed. Consequently, efforts to easily separate the fermentable sugars from these toxic compounds were not successful without significant concomittant losses of the fermentable sugars. While several detoxification methodologies were attempted, under the limited scope of this project, all possible detoxification treatments were not investigated. Unless a suitable detoxification procedure can be developed, the potential for conversion of organosolv spent liquor carbohydrates into liquid fuels by fermentation is very low.

This report is intended for those scientists and technical managers involved in developing and assessing the technology of the organosolv pulping process, and more particularly, to provide a better understanding of the nature and potential uses of the organosolv spent liquors.

ABSTRACT

The organosolv pulping process produces a spent liquor containing a high level of hemicellulose carbohydrates, primarily pentosan. The conversion of this potential waste stream into a marketable byproduct such as ethanol or butanediol by fermentation using the pentose-fermenting bacterium, Klebsiella pneumoniae would improve the economic attractiveness of organosolv over the conventional pulping processes.

A series of organosolv spent liquors, prepared under various process conditions, were characterized with respect to carbohydrate content (pentose, pentosan), lignin content, and their potential fermentability by K. pneumoniae to produce 2,3-butanediol and ethanol. The majority of the samples contained pentosans comprising 25 to 40% (w/v) of the total solids which ranged from 37 to 70% (w/v) of the spent liquors. Total lignin contents were less than 10% (w/v) of the total solids. Fermentation results indicated the presence of compounds which inhibited the growth and fermentation of K. pneumoniae as all butanediol yields, based on the available pentose, were less than 20% of the control yields.

Several attempts to remove the toxic compounds by standard procedures were unsuccessful. A combination of ion-exchange and gel filtration was used to separate a totally fermentable fraction of the spent liquor, however the remaining pentose in this fraction represented less than 10% of the original pentose present in the spent liquor. The unidentified toxic compounds were shown by HPLC analysis to be closely associated with sugar-degradation materials and not the lignin components present in the spent liquors.

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EXECUTIVE SUMMARY

Organosolv pulping processes produce a spent liquor waste stream which can contain high levels of hemicellulosic carbohydrates, the majority of which are pentose sugars. In order to improve the economics of the organosolv process the carbohydrates in the spent liquor should be utilized in the production of a marketable byproduct.

Because of the recent interest in biomass-to-liquid fuel fermentation processes which aim to make efficient use of the hemicellulose fraction by employing microorganisms capable of pentose fermentation, the technical feasibility of using the organosolv spent liquor as a source of fermentation substrate was studied. For this study, several organosolv spent liquors were prepared and supplied to Forintek Canada Corp. by Econotech Services Ltd. to be chemically characterized and tested for fermentability. This study tested the ability of the bacterium, Klebsiella pneumoniae, which can ferment pentose and other hemicellulosic carbohydrates into 2,3-butanediol and ethanol, to utilize organosolv spent liquor as a fermentation substrate. Other fermentations by different bacteria and yeast were tested by Econotech Services Ltd.

A total of 15 different organosolv spent liquor samples were analyzed for total solids, furfural, methanol, acetic acid, pentose, pentosan, Klason lignin, and acid-soluble lignin content. The majority of samples contained pentosans comprising 25 to 40% (w/v) of their total solids which ranged from 37 to 70% (w/v) of the spent liquors. Total lignin content was less than 10% (w/v) of the total solids. The chemical characterization

confirmed the high potential of the spent liquors to serve as fermentation substrates.

Fermentation results were not encouraging. In all cases, when there was no additional treatment of the spent liquors, butanediol yields obtained from the spent liquors at 2% (w/v) pentose concentration were less than 20% of the control yield obtained when 2% (w/v) xylose was used as the substrate. These results indicated the presence of compounds which were inhibitory to the fermentation and growth of K. pneumoniae. Similar inhibition of fermentation was observed for all of the microorganisms studied by Econotech Services Ltd.

Several attempts were made to remove the toxic compound(s) from the organosolv spent liquors. These studies comprised the majority of the effort made by Forintek Canada Corp. A totally fermentable substrate was obtained only after extensive ion-exchange and gel filtration treatments were used which unfortunately also removed the majority (greater than 90%) of the original pentose present in the spent liquors. Such a pretreatment step would not be economically feasible.

No attempt was made to identify the toxic compound(s), however it appears to be closely associated with the sugar-degradation products and not the lignin-derived components as shown by HPLC analysis.

Because of the inability of the organosolv spent liquors to serve as fermentation substrates the fermentation process development originally proposed as part of this project could not be carried out.

In the final analysis, in order for organosolv spent liquors to be used as fermentation substrates further research is necessary to define either a more selective detoxification treatment which does not remove the majority of the fermentable sugars or, more unlikely, organosolv process conditions which eliminate the production of the unknown toxic compound(s).

INTRODUCTION

The chemical processes which are of commercial importance in the pulping industry involve the solubilization and removal of the lignin fraction from wood pulp. Traditional pulping methods rely on two major lignin reactions, the sulfonation of lignin to water-soluble lignosulfonates in the sulfite processes, and hydrolysis of lignin to give alkali-soluble phenolate lignin in the kraft (alkaline) processes. The kraft process is the dominant pulping process today because of its comparative simplicity and rapidity of operation, as well as the valuable properties of the pulp produced (Rydholm, 1965). However, there are problems with these two popular processes which include the costly recovery of process chemicals and potential environmental hazards resulting from the chemicals released into the wastewater stream and air. A highly condensed lignin residue which cannot be removed by conventional extended cooking methods without any degradation of the carbohydrate fraction is also produced. This unreactive lignin has limited applications for by-product commercialization.

A variety of alternative methods have been studied for some time to optimize the removal of wood lignins using solvents other than water or alkali and to solubilize and separate the lignin in a relatively unmodified form. Alternative solvents which have been tested include alcohols such as methanol, ethanol, butanol, glycol and phenol (Aronovsky and Gortner, 1936), ethers such as dioxane (Engel and Wedekind, 1933) and polyglycol compounds (Grondal and Zenczak, 1956). These organic solvent treatments of wood require the presence of minute quantities of acid and water

to enhance delignification (Schuerch, 1957). The lignins dissolved in the spent liquor of these aqueous organic solvent (organosolv) treatments of wood retain most of their polymeric structure and can be precipitated from the liquor with water to be eventually sold as a by-product. Although the organosolv process appears to be inferior to conventional processes in terms of pulp quality and simplicity of operation (i.e. the flammable organic solvents require special caution in operation), it is considered to be less environmentally polluting since sulphite is not released in the waste stream, as occurs in the conventional processes. The organosolv process is also potentially more economically attractive as the lignin fraction can be separated in an unmodified form from the wood pulp and can then be easily recovered by precipitation with water from the organosolv spent liquor. Furthermore, the dissolved hemicellulose fraction in the spent liquor, which is not precipitated with the lignin, may be utilized as an inexpensive source of fermentable carbohydrates for liquid fuel production.

Recent research in our laboratory has demonstrated the technical feasibility of using a fermentative bacterium, Klebsiella pneumoniae, for the conversion of hemicellulose-derived sugars into potential liquid fuels and chemical feedstocks, viz. 2,3-butanediol and ethanol. This microorganism utilizes all major components of wood hemicellulose hydrolyzates, including hexoses (glucose, galactose, and mannose), pentoses (arabinose and xylose), disaccharides (cellobiose and xylobiose), uronic acids (glucuronic and galacturonic acids), as well as

acetyl groups common in hardwood hemicelluloses (Yu and Saddler, 1982a; Yu et al., 1984a). These substrates are converted into butanediol and ethanol at an efficiency which is close to the theoretical maximum of 0.5 g of product per gram of sugar utilized (Yu and Saddler 1983; Yu et al., 1984b). It is therefore of interest to investigate whether or not similar high yields of products could be achieved from the wood hemicelluloses obtained from the waste stream of the organosolv treated wood. A higher value byproduct from the hemicellulose component obtained from the organosolv pretreatment of wood could be crucial to the economic viability of any biomass conversion process or pulping process based on organosolv treatment.

A recent increase in interest in the commercial viability of the organosolv pulping process prompted the investigation by Econotech Services Limited, to examine the economic and technical feasibility of the organosolv systems, particularly the aqueous alcohols system, using methanol under acidic conditions. The hemicellulose fractions from their studies were examined by our laboratory for bacterial fermentability in the production of 2,3-butanediol. The potential uses of butanediol are extensive and resulted in earlier workers, in pre-petrochemical times, proposing a chemical industry based upon this compound (Long and Patrick, 1963). Initially, hemicelluloses in the spent liquor from the organosolv pretreatment process were tested for fermentability by Klebsiella pneumoniae for the production of butanediol. Unfortunately, the hemicellulose fractions, or the spent liquors, from the organosolv process were found to be

totally inhibitory to fermentation by K. pneumoniae. Due to the poor fermentability and potent inhibitory nature of the spent liquors, further work on the enzymatic hydrolysis or the combined hydrolysis and fermentation process could not be carried out until the inhibitory effects of the spent liquors were resolved.

It has long been recognised that there are inhibitory compounds present in the acid hydrolysates of wood (Leonard and Hajny, 1945; Hajny, 1981). Many compounds have been tested as inhibitors, such as lignin-derived phenolics and products from carbohydrate degradation (Lee and McCaskey, 1983). Methods used to treat hydrolysates to enhance their fermentability have only been developed empirically, as there is no detailed analysis methods for wood hydrolysis liquors (Hajny, 1981). Such detoxification methods include cation exchange, anion exchange, solvent extraction, charcoal treatment, molecular sieve treatment, overliming, gel filtration and adsorption, and mixed bed ion-exchange chromatography (Hajny, 1981; Clark and Mackie, 1984; Fein el al, 1984; Tran and Chambers, 1986). As the inhibitory compounds in the spent liquors from the organosolv treatments were thought to be similar to those obtained in traditional acid hydrolysates, the effectiveness of some of these detoxification methods on the spent liquors were investigated.

After considerable effort, which constituted a large portion of the work carried out in this study, a completely fermentable sugar fraction was obtained from the spent liquor after treatment by one of the detoxification methods. Some of the fermentative

inhibitory factors in the spent liquor were also isolated. It appears that the major inhibitory factors were not associated with the phenolic compounds and were primarily associated with the sugars or sugar degradation products. No further attempts were made to identify the inhibitors derived from the spent liquor samples.

It is apparent that any future use of the hemicellulose rich fraction obtained as a byproduct of organosolv pulping will have to first resolve the high level of inhibitory products associated with this stream. As it is unlikely that conditions could be defined which would eliminate the production of these degradation products, future research should focus on the development of an effective, inexpensive detoxification method or the identification of a suitable microorganism capable of fermenting the hemicellulose sugars into a marketable product despite the presence of these toxic compounds.

OBJECTIVES

The objectives of this project were: (i) to chemically characterize the organosolv spent liquor samples provided by Econotech Services Limited to obtain quantitative and qualitative analysis of the hemicellulose carbohydrates and lignin compounds present, (ii) to demonstrate the feasibility of using the spent liquors as fermentation substrates for the production of butanediol and ethanol by the bacterium, Klebsiella pneumoniae, and (iii) from the results obtained, provide input for future research direction for the utilization of organosolv spent liquors.

METHODS

Hemicellulose Substrates:

Fifteen samples were supplied by Econotech Services Ltd. during the contract year (Oct., 1985 to Oct., 1986). The samples were:

1. Pooled spent liquors from cooks No. 10, 11, 12 (received Oct., 1985);
2. Pooled spent liquors from cooks No. 13, 14, 15, 16 (received Oct., 1985)
3. Spent liquor from cook No. 17 (received Oct., 1985);
4. Spent liquor from cook No. 18 (received Oct., 1985);
5. Spent liquor from cook No. 19 (received Nov., 1985);
6. Spent liquor from cook No. 20 (received Nov., 1985);
7. Spent liquor from cook No. 21 (received Nov., 1985);
8. Spent liquor from cook No. 22 (received Dec., 1985);

9. Spent liquor from cook No. 23 (received Jan., 1986);

10. Spent liquor from cook No. 24 (received Jan., 1986);

11. Spent liquor from cook No. 25 (received Feb., 1986);

12. Spent liquor from cook No. 26 (received Mar., 1986);

13. Spent liquor from cook No. 29 (received Mar., 1986);

14. Spent liquor from cook No. 30 (received May, 1986);

15. Spent liquor from cook No. 31 (received May, 1986).

Except for cooks No. 29, 30 and 31, all samples were accompanied by the conditions of each sample treatment, and analytical data. All samples as received were cold but not frozen. Cooks No. 17 and 18 were received at room temperature.

Some samples were received in very dilute form and therefore necessitated further concentration at Forintek to a syrup of around 20% solids content using rotary evaporation at 50 °C under high vacuum.

Neutralization and Detoxification of the Samples:

Preliminary studies on detoxification of the samples were carried out by an ion-exchange treatment alone or in combination with the overliming procedure. Overliming was performed by raising the pH of the samples to around 10.0 with lime (calcium oxide, or calcium hydroxide), filtering to remove excess sulfate (calcium sulfate), then lowering the pH of the filtrates to 4.0 using phosphoric acid, filtering to remove excess calcium (calcium phosphate), and finally adjusting the sample pH to the desired level for fermentation (pH 6.5) using potassium hydroxide.

Ion-exchange treatment involved the elution of samples with water through the weakly basic anion exchange resin, Amberlite IR-45, or the mixed bed resin Amberlite MB-1, or BioRad AG 501-X8, or the strongly acidic cation exchanger, Amberlite IR-120. Anion exchange treatment of the samples with Amberlite IR-45 was carried out either batchwise by mixing the sample with a predetermined amount of Amberlite IR-45 (as calculated by the total exchange capacity in meq./ml wet resin) for 60 minutes at room temperature, or by passing the sample through a glass column filled with predetermined amounts of the resins at a particular flow rate to achieve the pH adjustment. Mixed bed ion-exchange treatment of the samples with Amberlite MB-1 or the Biorad AG-501-X8 was carried out by passing the sample through a glass column filled with predetermined amounts of resin at two resin-to-sample solids (w/w) ratios, 7.2:1 (normally used in such treatment) and 3.6:1 (to minimize sugar loss). The cation exchange resin, Amberlite IR-120, was used in series with the anion exchanger, Amberlite IR-45. The two glass columns were charged with the individual resin and connected in series with a small diameter rubber tubing leading from the bottom of the cation exchange resin column to the top of the anion exchange column. The second column was about four to six inches below that of the cation exchange column. The sample was eluted with water through both columns at a flow rate of 0.1 to 0.2 bed volumes per minute. At each ion-exchange treatment, five fractions were collected, each with the same volume as the volume of sample applied. The fractions containing the highest pentose

concentrations were then pooled and rotary evaporated to a more concentrated form prior to use in the butanediol fermentation process.

An alternative method of removing the fermentation inhibitors was by separating of the inhibitory factors from the fermentable sugar fraction of the organosolv spent liquor by gel filtration and adsorption using Sephadex LH-20 (Pharmacia). Sample cook No. 23 was assayed by column chromatography with water at a bed volume-to-sample volume ratio of 10:1 and a flow rate of 2 to 3 ml per minute, collecting 10 to 20 ml fractions until twice the bed volume had passed through the column. Sample cook No. 24 was also run on a chromatograph using water eluant but at a bed volume-to-sample volume ratio of 22:1 and a flow rate of 0.5 to 1.0 ml per minute, collecting 5 to 10 ml fractions until three bed volumes had passed through the column. The adsorbed material was finally eluted from the column with two bed volumes of 95% ethanol. As Sephadex LH-20 is unstable at acid pH, the samples were treated batchwise with Amberlite IR-45 prior to their application on the column. The fractions collected were scanned at 520nm on a Spectronic 710 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) and analyzed for total pentose by the p-bromoaniline method (Deschatelets and Yu, 1986). The fractions with highest absorbance at 520nm (i.e. pentose concentration) were pooled. The pooled fractions were either rotary evaporated or freeze dried to a concentrated form prior to use in the butanediol fermentation process.

Untreated control samples were tested for their inhibitory

effect when they were added as a supplement to medium containing 2% (w/v) xylose for butanediol fermentation under established conditions, as detailed in a later section. All treated samples were used at pentose levels of 2% or 5% (w/v) and a typical butanediol fermentation was carried out.

Fermentative Microorganism and Medium:

Butanediol fermentation of hemicellulose-derived sugars were studied using Klebsiella pneumoniae (formerly Aerobacter aerogenes NRRL B-199; Klebsiella oxytoca ATCC 8724) obtained from the National Research Council of Canada Culture Collection (strain NRCC 3006). Cultures were maintained on nutrient agar slants at -20 °C.

Fermentation studies were carried out in a chemically defined medium (Kp2 medium) as shown in Table 1 (Yu and Saddler, 1982). All medium ingredients except acetic acid and substrate were prepared together with pH set at 6.5, and sterilized by autoclaving at 121 °C for 15 minutes. All substrates were prepared separately at the desired concentration, sterilized by autoclaving, and then transferred aseptically to presterilized medium to the appropriate final concentration. Acetic acid was prepared as a 5% (w/v) aqueous solution at pH 6.5 (adjusted with KOH), and autoclaved separately prior to its addition (if necessary) to the medium to achieve a final acetate concentration of 0.5% (w/v) before inoculation of bacteria. Medium for inoculum preparation was prepared in Erlenmyer flasks (10 ml in 50 ml flasks) for aerobic cultures. Medium for fermentation studies

under finite air conditions was prepared in Wheaton serum bottles (10 ml in 60 ml vials) and sealed with butyl rubber stoppers and aluminum caps.

When spent liquor samples, with or without detoxification treatment, were used as substrates for fermentation, the samples were prepared and autoclaved with known pentose concentrations. Appropriate aliquots of the autoclaved samples were transferred aseptically to presterilized medium ingredients to achieve a final substrate concentration of 2 or 5% (w/v). Acetic acid was added whenever required to achieve a final acetate level of 0.5% (w/v). Cultures were incubated under finite air conditions at 30°C with shaking (150 rpm) for 4 days and sampled on alternate days during incubation.

Culture Conditions:

Working cultures of K. pneumoniae at -20°C were thawed at 37°C for 30 minutes and used to inoculate medium broths containing glucose at 1% (w/v). The cultures were incubated aerobically at 37°C with shaking (150 rpm) for 24 hr, and then used to inoculate fresh test media (routinely with 2% (v/v) inoculum). The test cultures were incubated at 30°C under finite air conditions with shaking (150 rpm) for specified time periods.

Acclimatization of cells to high initial sugar concentrations was carried out by serial subculturing of cells grown in media containing increasing concentrations of sugars under aerobic conditions. The optimal scheme adopted involved daily subculturing of K. pneumoniae under aerobic conditions

through a stepwise increase in the levels of xylose (1%-5%-10%-15% (w/v) (Yu and Saddler, 1983). The resulting cultures were used as the inocula in fermentation media with high substrate or inhibitor concentrations. Alternatively, the acclimatized cultures were maintained at -20°C and when needed, the acclimatized cultures were thawed and transferred to fresh medium containing 15% (w/v) xylose in the absence of added acetic acid and incubated aerobically at 37°C for one day. The cultures were then aseptically centrifuged at 10,000 x g for 10 minutes, resuspended in sterile distilled water to the original volume, and then used to inoculate fresh test media (2% (v/v) inoculum). When resting cells of the acclimatized K. pneumoniae were used, the frozen acclimatized cultures were thawed, transferred to fresh medium with 15% (w/v) xylose in the absence of added acetic acid and incubated aerobically at 37°C for one day. The cultures were then aseptically centrifuged at 10,000 x g for 10 minutes, washed twice with sodium acetate buffer (0.05 M acetate, pH 6.0) of the same original volume, and finally resuspended in the original volume of presterilized test medium containing the various substrates.

At appropriate times during incubation, the bacterial cultures were sampled and harvested by centrifuging (4°C) at 10,000 x g for 15 minutes and the supernatant fluids were analyzed. Alternatively, cultures were harvested using a Fisher Microcentrifuge Model 235A (Fisher Scientific Co.). Culture supernatant fluids were then analyzed for sugar utilization and solvent production.

Analytical Methods:

Pentosans were analyzed by the method outlined in TAPPI standard T223-os-71. Klason lignin determinations were carried out as per TAPPI standard T222-os-75. Acid soluble lignin in the filtrate from the Klason lignin analysis was quantitated by absorbance reading at 212.5 nm, using a fractionated sample of aspen milled wood lignin as standard. Hexosans were analyzed by the anthrone reaction (Shields and Burnett, 1960).

Volatile fermentation products were analyzed by gas chromatography (Ackman, 1972) with a Varian model 4600 gas chromatograph equipped with an autosampler and a Varian 401 data system. A Chromosorb 101 column (6 ft x 1/8 in., 100-120 mesh; Chromatographic Specialties Ltd.) was used with a flame ionization detector. The injection and detector temperatures were set at 160 °C and 250 °C, respectively. The column oven temperature was operated at 160 °C for 2 minutes, raised to 190 °C in the next minute, and then held for an additional 7 minutes. Helium, saturated with formic acid vapor, was used as a carrier gas and the flow rate was set at 30 ml/min. Quantitation was performed by external standard calibration.

Glucose was determined colorimetrically by the glucostat enzyme assay (Raabo and Terkildsen, 1960). Pentose was analyzed by the method initially proposed by Roe and Rice, 1948 and modified by Deschatelets and Yu, 1986 for this study. The method is based on the formation of furfural from pentoses in acetic

acid containing thiourea (an anti-oxidant) at 70°C and the reaction of furfural with p-bromoaniline acetate to form a pink colored product. In the routine assay, 1.0 ml of p-bromoaniline reagent (p-bromoaniline in acetic acid saturated with thiourea) was added to 0.2 ml of sample and standard solutions for both the tests and the corresponding blanks. The purpose of the blanks was to correct for furfural already present in the sample as well as for any possible coloration associated with the sample. The tests and blanks were prepared in an identical manner, except that all tests were placed in a 70°C water bath for 10 minutes while all blanks were incubated in the dark at room temperature. The tests were rapidly cooled and then incubated in the dark for 70 minutes at room temperature. The absorbance of all tests and blanks was read at 520 nm with a DMS-90 Spectrophotometer (Varian Associates, Inc., Palo Alta, Calif.). D-xylose was used as the standard.

Individual sugars (monosaccharides and disaccharides) were analyzed by High Performance Liquid Chromatography (HPLC) using a Varian Model 5000 Liquid Chromatograph interfaced with autosampler and Varian CDS 401 data system and a BioRad HPLC carbohydrate analysis column (300 x 7.8 mm Aminex HPX-87P, heavy metal form) and a reflective index (R.I.) detector (Wentz et al, 1982). The column temperature was 85°C and the solvent (degassed double distilled water) flow rate was 0.6 ml per minute. The column was preceded by a cationic exchanger (BioRad Micro-Guard Cation H cartridge, 40 x 4.6 mm), and an anionic exchanger (BioRad Micro-Guard Deashing Anion, 40 x 4.6 mm) for improved

performance and durability of the analytical column. Quantitative analysis of the samples was carried out either by using erythritol as an internal standard or with external standards. This enabled the detected sugar levels to be corrected for sugar losses during the pretreatment steps.

RESULTS AND DISCUSSION

Modifications of Pentose Assay

In the past, sugar determination was carried out colorimetrically by reducing sugars analysis, using reagents such as dinitrosalicylic acid (DNS)(Miller, 1959) or arseno-molybdate (Nelson, 1944; Somogyi, 1952). Although both methods are simple and rapid, the assays are not suitable for quantitation of pentoses in the presence of other sugars (hexoses and oligosaccharides) and reducing equivalents such as uronic acids which are common in the hemicellulose stream of the organosolv process. Attempts were therefore made to establish an interference-free assay procedure specific for xylose. The p-bromoaniline assay method adopted was tested against common non-pentose components known to be present in biomass hemicellulose hydrolysates in order to assess the potential for interference in this pentose assay (Table 2). These include major hexoses, methyl pentoses, uronic acids, and acetic acid. The highest error of estimation obtained from all the components tested was less than 5.5%. The assay was also tested against lignin (obtained from steam exploded aspen), the hemicellulose fraction and the Kp2 medium (as detailed in Table 1) used in the butanediol fermentation process. Although slight changes in the intensity of colour formation were observed, the linearity of the D-xylose standard curve was not affected, resulting in less than 6% error when the sugars were quantified (data not shown). This assay method was used routinely for monitoring the pentose sugars in the organosolv samples during sample detoxification and

fermentation processes.

Chemical Analysis of Spent Liquor Samples

Two of the spent liquor samples received from Econotech Services Limited were the pooled liquors from cooks No.10, 11, 12, and cook No. 13, 14, 15, 16. Both samples were very dilute and preliminary concentration by rotary evaporation at 50 °C under high vacuum was performed until a solids content of 20 to 30%(w/v) was reached. Chemical characterization of the concentrated samples is summarized in Table 3. When a 2% (w/v) xylose solution was supplemented with a 1% (v/v) solution of either of these pooled fractions less than 20% of the normal butanediol yields were obtained after 4 days growth as shown in Table 4. Although the samples were concentrated, there were not enough pentose and pentosans in the concentrated samples to allow us to carry out further fermentation studies. We were also unable to carry out any systematic detoxification studies as there was not enough sample provided. From the data provided by Econotech Services Limited (Table 5), it was noted that the pretreatment process generating the spent liquors of cook No.10, 11, 12 and cook No.13, 14, 15, 16 was different from before, in that, $MgSO_4$ (0.024 M and 0.05 M respectively) was used as the catalyst in the organosolv process. It is possible that this procedure was not as effective in separating out the hemicellulose fraction from the wood pulp.

Chemical characterization of the next batch of spent liquor samples which included cooks No. 17, 18, 19, 20, 21, and 22 is summarized in Table 6. The results indicate the presence of higher levels of carbohydrates in these samples with the highest pentosan

levels detected in cook No. 17 and lowest in cook No. 22. However, a reverse trend was observed for the total pentose level as assayed by the p-bromoaniline method. A review of the process technical data in Table 5 indicated that cook No. 17 was cooked at a higher pH, and for a longer time, than cook No. 22. There might be more acid hydrolysis of the wood substrate during the pretreatment process of cook No. 22 than cook No. 17, hence more pentose was detected resulting in correspondingly lower pentosan levels in the spent liquor of cook No. 22. Generally, the non-carbohydrate content of these samples was quite high with the majority of the material detected as lignin, constituting about 10% (w/v) of the total fraction. Although these samples had been precipitated with water to remove lignin (by Econotech Services Limited), there was still a considerable amount of lignin detected in these samples, with about 60 to 70% of the total lignin content being present in the acid-soluble form. It is probable that this material is highly inhibitory to fermentative microorganims such as K. pneumoniae.

Some of the spent liquor samples contained considerable amounts of acetic acid and consequently did not require addition of acetic acid to the fermentation medium as described in the Materials and Methods section. The glucose levels in these samples were generally low (less than 0.25% w/v), with small quantities of furfural (around 0.01% w/v) still detected in the original samples. About 50 to 60% of the sample fraction was not identified. This unidentified material probably included degradation products from sugar and lignin, as well as uronic acids and acetate common in hardwood hemicellulose.

Each of the pooled organosolv samples was adjusted to either a 2% or 5% pentose content and then added to a typical 2% xylose substrate used for butanediol production by K. pneumoniae. The control was the 2% xylose substrate which was not supplemented by any of the organosolv fractions. The results shown in Table 7 indicated the same trend as found with the previously undetoxified samples. An inhibition of over 80% was observed on the butanediol production when compared to the control of xylose with no sample supplement. The 5% supplement concentration resulted in even higher inhibition with values less than 10-20% of the control. It was apparent that growth as well as butanediol production was inhibited as there was little or no increase in the optical density of the culture (data not shown).

Chemical characterization of the last batch of organosolv spent liquor samples, which included cooks No. 23, 24, 25, 26, 29, 30, and 31, are detailed in Table 8. The pentosan levels of cook No. 30 and 31 could not be quantitified due to the extremely hygroscopic nature of the samples. These samples contained the highest levels of lignin and comparatively lower pentose levels. Cook No. 29 had the lowest levels of pentose and pentosan. This batch of samples contained less methanol than the previous samples and they all contained small quantities of acetic acid, with cook No. 23 and 24 having the higher amounts. Trace quantities of furfural and glucose were also detected in these samples. The solids content of these samples was slightly higher than previous samples. Most of these samples contained considerable amounts of pentose sugars and were tested for their potential inhibitory

effect. When a 2% pentose concentration of the undetoxified samples were supplemented to a typical xylose fermentation, less than 10% of the typical butanediol yields were obtained (Table 9).

Detoxification by the Ion-exchange Method

As the unhydrolyzed samples already contained high levels of monosaccharides they were treated with ion-exchange resin (Amberlite IR-45) and then used directly as substrates for butanediol production. The detailed chromatographic data for this neutralization and detoxification method is presented in Table 10. Although the volume of resin used for the ion-exchange chromatography was calculated according to the total exchange capacity of the resin in meq. per ml wet resin (Amberlite Ion-Exchange Resins Laboratory guide, 1974), with the assumption that the total volume of sample applied was all acid anions, the ratio of resin-to-sample solids (w/w) was generally very low. When the fractions collected from Amberlite IR-45 chromatographed samples were assayed for pentose content there was virtually no sugar loss detected. The fractions containing the most pentose were pooled and concentrated to the same pentose concentrations present in the original fractions. A typical butanediol fermentation was carried out using a 2% (w/v) pentose concentration of the treated samples. From the data illustrated in Figure 1, the fermentability of the IR-45 treated samples was only partially restored with generally less than 10% solvents produced as compared to that of the control containing 2% xylose.

As treatment with the anion exchange resin Amberlite IR-45 failed to relieve the inhibitory effect of the samples, when used

at pentose sugar levels of 2% (w/v), a mixed bed ion-exchange resin using Amberlite MB-1 and BioRad AG-501-X8, was tested as an alternative method of detoxifying the samples. As indicated in Figure 2, both mixed bed ion-exchange treatments were more effective in restoring the fermentability of sample cooks No. 17 and 18 than the anion exchange method using Amberlite IR-45 (Figure 1). However, sugar losses resulting from both types of resin treatments were unacceptably high.

Approximately 50-70% of the original pentose present in sample cooks No. 17 and 18 were lost during treatment by Amberlite MB-1 while treatment with AG-501-X8 resulted in the loss of 86-98% of the original sugars. Further studies were not carried out on these fractions due to the lack of sample.

As the sugar loss by the AG-501-X8 treatment was unacceptably high, sample cook No. 19 was eluted through MB-1 instead of AG-501-X8, using the same elution flow rate and the same high ratio of resin-to-sample solids of 7.2:1 used in previous treatments. The fractions collected after elution from the column had less than 2% of the original sugar (Table 11). Cook No. 20 was then passed through an MB-1 column with a reduced resin-to-sample solids ratio of 3.6 (w/w), keeping the same elution flow rate constant throughout these studies. A sugar recovery of 51.4% was obtained after this treatment. This treatment of cook No. 20 seemed to indicate that reducing the ratio of resin-to-sample solids lowered the loss of pentose sugars by 50%. Consequently, cook No. 21 was eluted through AG-501-X8 with a lower ratio of resin-to-sample solids of 3.6:1 instead of the higher ratio of 7.2:1 used previously. Although

more than 50% of the original sugar was recovered the samples still contained inhibitory compounds that effectively prevented butanediol production (Figure 2). The highest butanediol level obtained from fermentation of the treated samples was less than 40% of the control value obtained with an equivalent amount of xylose. The growth of the cultures was also inhibited as there were very little increase in the optical density of the cultures.

Use of Resting Cells to Circumvent Growth Inhibition of the Fermentative Microorganism

As the growth of the cultures was also inhibited, it was postulated that low butanediol production could be an indirect result of poor cell growth. As other work had shown that resting (non-growing) cells of Klebsiella pneumoniae could tolerate higher levels of inhibitory materials (Yu et al., 1985), a similar approach was taken in an attempt to overcome the inhibitor problems associated with the organosolv liquors. By using a large inoculum of resting cells instead of the normal growing cells (refer to culture conditions in Methods section), butanediol yields were partially restored as shown in Table 12. However, butanediol yields were still usually less than 20% of the control yield (data not shown) indicating that inhibition of cell growth was not the only factor operating in the fermentation inhibition.

Detoxification by a Combined Ion-exchange and Overliming Method

Our initial detoxification methods for treating the organosolv samples had involved a single treatment with various resins. This proved to be very ineffective in removing the

fermentative inhibitors. Based on studies presented in earlier reports, overliming (differential precipitation of samples via pH shifts using $Ca(OH)_2$ and H_3PO_4), seemed promising in that less than 20% inhibition was observed in the cultures supplemented with overlimed organosolv samples. As our previous work discussed earlier had shown there was less sugar loss after anion exchange (Amberlite IR-45) treatment, it was used in a sequential manner with overliming. The results obtained after treatment of samples No. 19, 20 and 21 are indicated in Figure 3. The inhibitory effect of the samples on butanediol fermentation was reduced considerably by combining the anion exchange and overliming treatments. The final butanediol levels obtained from fermentation of the treated samples ranged from 35% (for cook No. 21) to 65% (for cook No. 19) of the control values. Ethanol production was also enhanced indicating that there seemed to be a switch in the metabolic pathway of the fermentative organism when it was fermented under different culture conditions. The net result therefore was a substantially higher total solvent (butanediol and ethanol) levels. These values approached the corresponding levels obtained with the control xylose fermentation. However, the butanediol-to-ethanol ratios of solvents obtained as a result of fermentation of the hemicellulose samples were all around 1:1 (w/w) whereas the corresponding ratio of the control xylose fermentation was around 11:1 (w/w).

Samples from cook No. 23, 24, 25, 26, 30 and 31 were also treated sequentially with an anion exchange resin (Amberlite IR-

45) followed by overliming. The data in Figure 4 showed there was very low butanediol production with little or no sugar utilization by the bacteria. It appears that the combined treatments of ion-exchange and overliming were ineffective for these spent liquor samples. This could be due to variations in the pretreatment methods used to prepare these samples. As shown by the technical data supplied by Econotech (Table 5), these five samples were cooked at a lower final pH in the cooking liquor than the previous samples. The spent liquors were also treated with lower volumes of water to enhance the removal of lignin. Our chemical analysis revealed that most of these samples generally had a higher lignin content (Table 8).

As detoxification of the spent liquor samples proved to be ineffective when a combination of anion exchanger and overliming treatments was used, an alternative method involving a mixed bed ion-exchange resin was attempted. Earlier studies (Figure 2) had shown that there were promising detoxification effects as demonstrated by treatment of the samples with mixed bed ion-exchange chromatography, but there were unacceptable losses of the sample pentose sugars during this treatment. By using the more conventional approach and optimized elution conditions, the spent liquors from cook 30 and 31 were deionized by sequential treatment with a cation (Amberlite IR-120(H)) and an anion exchange resin (Amberlite IR-45). Treatment with the strongly acidic cation exchanger, IR-120(H), resulted in the removal of the cations while the anionic exchanger, IR-45, removed all the acid anions in the spent liquor samples.

As a result, the cation and anion exchanged samples lost

about 25% of the pentose sugars present in the original samples as opposed to the 40 to 90% loss observed in our earlier detoxification studies. However, no significant improvement was observed in the butanediol production when 2% pentose from the deionized samples was fermented. As the data in Table 12 shows, neither the incorporation of the overliming procedure to the mixed bed ion-exchanged samples, nor the use of the non-growing (resting) cells of the K. pneumoniae, improved the butanediol fermentation results, despite the fact that these treatments had shown in our earlier studies to be effective in circumventing some of the inhibitory effect. The disappointingly low butanediol production might be due to the fact that the carbohydrate content of the samples after ion-exchange treatment was low and required considerable concentration to achieve sufficiently high sugar levels for subsequent fermentation. This probably resulted in the accumulation of toxic components in the samples which were not removed by the detoxification treatment, thereby preventing efficient butanediol fermentation.

Due to the poor fermentability and toxic nature of the spent liquor samples, proposed further studies on the combined hydrolysis and fermentation process without resolving the inhibitor problem would prove meaningless. Although much effort was expended to detoxify the spent liquor samples via ion-exchange chromatography and overliming, the fermentability of these samples was not significantly improved. The inhibitory effect may not have been caused by the cationic and anionic impurities from the organosolv pretreatment process generating

these samples. The inhibition was most probably caused by the carbohydrate or lignin degradation products present in the spent liquor samples resulting from the pretreatment process. An alternative approach to this problem of detoxification was to separate the inhibitory factors present in the spent liquor samples by gel filtration and adsorption methods.

Separation of Inhibitors by Gel Filtration and Adsorption

Sephadex LH-20 is a bead-formed, dextran gel with lipophilic (hydrophobic) properties and an affinity for aromatic and cyclic compounds which are the typical structures of phenolic derivatives expected from lignin degradation. Since LH-20 is not stable in strongly acidic solvents, the spent liquor was treated with Amberlite IR-45 to remove acid anions prior to elution through LH-20. Since sample cook No. 23 contained about 10% pentose sugars and as the previous detoxification methods showed only a slight improvement, further detoxification of this sample was attempted using Sephadex LH-20.

The spent liquor sample from cook No.23 was treated batchwise with Amberlite IR-45 until the final pH of the sample was neutral. At that point a lot of precipitate was present which was possibly the acid-soluble lignin in the sample which precipitated out at the high pH. The Amberlite IR-45 treated sample had to be filtered several times to remove the precipitate. The treated sample was applied on the Sephadex LH-20 column at a bed volume-to-sample volume ratio of 10:1 and a fast flow rate of 2 to 3 ml per minute. The elution profile of sample cook No.23 is presented in Figure 5. The IR-45 treated sample was

eluted through the column with water and after 2 to 3 bed volumes of water had passed through and had been collected in small volume fractions, the column was finally washed with 95% ethanol to regenerate the gel.

From the data illustrated in Figure 5, the sample pentose sugars were separated into the middle fractions (F4 to F8), whereas the darkest coloured and the most acidic components of the sample separated into the initial few fractions (F1 to F3). Three pooled fractions were collected with the first ,FD1 (fractions F1 to F4), containing the darkest coloured fractions in which little or no pentose sugar was detected. The second, FD2 (fractions F5 to F8), contained the highest pentose sugar levels, while the third, FD3 (fractions F8 to F20), which contained all the remaining fractions, contained no pentose sugars. The total amount of pentose sugar loss was 23% of the original amount applied to the column. The pooled fractions were then used to supplement xylose fermentation with the results shown in Table 13.

With no concentration step performed, a portion of the fractions F5 and F6 (prior to pooling into FD2) were fermented at a 1% pentose sugar level resulting in final butanediol levels of 55% and 35% of the control level obtained from 1% xylose fermentation. This seemed to show that the inhibitory factor in the sample was not removed completely from the sugar fraction. As little as 1% pentose sugar in the fermentation broth was not utilized totally for the production of solvents compared to that of the control. The remaining portions of these fractions were pooled into FD2 and concentrated to a 2% pentose sugar level

which was used as a supplement to a butanediol fermentation system with 2% xylose as the substrate. This procedure was to test the concentrated effect of inhibitors which were not removed. Partial inhibition was observed when a final butanediol level of 51% of the control level was reached. Increasing the amount of FD2 supplemented to the xylose fermentation system from 2.5 to 7.5% (v/v) only increased the inhibition by 5% (Table 13). This suggested that the inhibitory effect is not additive.

The significantly improved butanediol fermentation observed (as compared to previous findings using other detoxification methods) could also be due to the separation of the fermentative inhibitors into the different fractions before and after the sugar fraction. It is possible that any synergistic effect of the fermentative inhibitors was thereby minimized. To test this possibility the inhibitory effect of the fractions before and after the sugar fraction were also tested (Table 13). Butanediol fermentation on xylose was less inhibited when high concentrations of these two fractions were added as supplements to xylose fermentation, resulting in final butanediol levels of 71% and 59% of the control level respectively for FD1 and FD3. Hence, the inhibitory factors in these organosolv spent liquor samples must be closely associated with sugars or degradation products of sugars. Preliminary HPLC analysis of the sugar fractions obtained from elution through Sephadex LH-20 (Table 14) indicated the presence of three unknown peaks in addition to the two major sugar peaks of xylose and xylobiose. These latter sugars were the major components in the spent liquor samples. As

the unknown peak with the retention time of 16.4 min was present predominently in the later sugar fractions (pooled into FD2), it is possible that the fermentative inhibition was due to an unidentified compound which eluted at this retention time. As there was not enough sample from cook No.23 to repeat this treatment, another sample was used in subsequent studies to refine this detoxification method.

Since butanediol fermentation had been shown to be significantly improved when the spent liquor sample was eluted with Sephadex LH-20, this method was repeated on sample cook No.24, which had been cooked at the same temperature as cook No. 23 but for a longer time. This material contained more pentose sugars than cook No.23. The sample was deacidified with Amberlite IR-45 batchwise prior to elution through Sephadex LH-20. The flow rate was much slower (0.5 to 1.0 ml/min) than previously used and the ratio of bed volume-to-sample volume was increased to 22:1, to ensure adequate separation of the components in the samples. The elution profile of the Sephadex LH-20 treated sample cook No.24 is presented in Figure 6. After two bed volumes of water had passed through the column, 95% ethanol was used to elute the adsorbed material which was also collected.

Unlike the elution profile of sample cook No.23, cook No.24 was separated into a distinct pentose sugar peak and two coloured peaks. The first peak was from the acidic, darkest-coloured fractions, followed closely by the peak from the pentose sugars fractions which showed another slight peak. This was a shoulder peak which coincided with the next coloured peak from the less darkly-coloured fractions. Three pooled fractions were ultimately

obtained from fractions F1 to F7 (FD1), fractions F8 to F13 (FD2) and finally all the remaining fractions from F14 onwards (FD3). All three pooled fractions were freeze-dried and then reconstituted to the same volume as the sugar fraction (FD2). The ethanol extracted fractions, which would contain the aromatic compounds (lignin and lignin derivatives) of the spent liquor sample, were also pooled and rotary evaporated to dryness. Except for FD2, all the pooled fractions were used as supplements in the butanediol fermentation system using 2% xylose as the substrate. FD2 was fermented directly by K. pneumoniae at a 2% pentose sugar level. The results of the fermentations are shown in Table 15.

When FD2 was fermented at a 2% pentose sugar level, a final butanediol level of slightly over 100% of the control level was obtained although the rate of solvent produced, determined after 2 and 4 days incubation, was slower than the control. The same volumes of FD1 and FD3 as used in the FD2 fermentation (40% (v/v)) were used to supplement a 2% xylose fermentation. There was no apparent inhibition exerted by FD1 which resulted in a final butanediol yield and rate of solvent production similar to that of the control. However, partial inhibition was exhibited by FD3 as a final butanediol level of 42% of the control level was observed. There was less inhibitory effect observed when only 10% (v/v) FD3 supplement was used. Again, this showed that the inhibitory effect is not additive. When FD4 was used to supplement 2% xylose fermentation, the final butanediol yield was 15% lower than the control. It seemed that the inhibition of butanediol fermentation is only partially and less severely

caused by the phenolic compounds in the ethanol extract. The major inhibition was observed in the fraction closely associated with the sugar fraction. Detailed HPLC analysis indicated the presence of the same three unknown compound peaks in the FD3 fractions. The HPLC chromatographic profile of the individual representative fractions are illustrated in Figure 7.

As the different fractions contained various concentrations of sugars, they were chromatographed at different attenuation levels. The chromatogram from F4 and F6 were representative of the components in FD1 and there were only trace amounts of xylobiose (11.41 min) present along with some glucose (12.45 min) and an unidentified compound at a retention time of 13.03 minutes. The unknown in this fraction did not exhibit inhibitory behaviour (as shown in Table 15). As the fractions, F8, F10 and F12, began to show the presence of xylose (13.49 min), there also appeared the two predominant peaks at 13.06 and 13.92 min retention time. However, they were not factors in the inhibition of butanediol fermentation as the fermentation of 2% pentose sugars from FD2 (which included F8, F10, F12) resulted in solvent production slightly higher than the control, accompanied by total pentose utilization. Moreover, the unknown compound peak at 16.54 min retention time was predominantly present in the later fractions representative of FD2 and in the fractions representative of FD3. Since the major inhibition of the butanediol fermentation system was observed in the xylose fermentation supplemented with FD3, it appears that the fermentative inhibition was caused by this unknown compound peak at 16.54 min retention time.

Although Sephadex LH-20 could effectively separate the fermentative inhibitors in the spent liquor sample, giving a completely fermentable sugar fraction, the total loss of pentose sugars after the detoxificaion treatment was 98% of the pentose orginally present in the sample. Of this loss, 53% occurred during the treatment with Amberlite IR-45 while 45% occurred as a result of the gel filtration step. Economically, this method of detoxifying the spent liquor samples is obviously not feasible. Furthermore, as evidenced by HPLC analysis, these fermentation inhibitors may possibly be unknown compounds associated with the fermentable sugars.

CCONCLUSIONS AND RECOMMENDATIONS

The inability of K. pneumoniae to ferment the untreated spent liquors from the organosolv process has clearly demonstrated the presence of compounds which are inhibitory to the normal biochemical processes of this microorganism. The results of this fermentation study confirm those reported by Econotech Services Limited which demonstrated inhibition of fermentation by other microorganisms.

The inhibitory compound(s) appear to be closely associated with the carbohydrates in the spent liquors and are most probably carbohydrate degradation products formed during the organosolv process.

Removal of these inhibitory compounds was shown to be technically feasible by means of gel filtration and adsorption with a hydrophobic gel such as Sephadex LH-20. However, such detoxification of the spent liquors is not economically practical due to the substantial loss of fermentable pentose sugar which occurs concurrently with the removal of the inhibitory fractions.

Practical use of the organosolv spent liquor as a fermentation substrate would only be feasible if a more selective and inexpensive detoxification procedure is developed such that the maximum recovery of fermentable carbohydrates is obtained. Another possibility is that the organosolv process conditions could be defined such that it would minimize the formation of these inibitory carbohydrate degradation products thereby eliminating their presence in the spent liquor. If fermentation of the hemicellulose sugars in the spent liquor is to remain a viable option for organosolv byproduct credits, one or both of the above possibilities should be further explored.

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Table 1. Composition of Kp2 Medium:

Ingredients	per liter
K_2HPO_4	8.7 g
KH_2PO_4	1.5 g
$(NH_4)_2SO_4$	3.0 g
MES (2-(N-morpholino)ethanesulfonic acid)	10.0 g
Glacial acetic acid	5.0 g
EDTA	0.4 g
$MgSO_4 \cdot 7H_2O$	240.0 mg
NaCl	100.0 mg
$CaCl_2 \cdot 2H_2O$	14.0 mg
$FeSO_4 \cdot 7H_2O$	10.0 mg
$MnSO_4 \cdot 4H_2O$	2.8 mg
$ZnSO_4 \cdot 7H_2O$	7.5 mg

Table 2. Potential Interference of p-Bromoaniline Assay by Non-pentose Components Present in the Biomass Hydrolyzates.

Biomass component (0.5 g/L)	Assay on component[a] (no D-xylose)		Assay on component[b] plus D-xylose	
	Absorbance	Xylose equivalents (g/L)	Net Absorbance	Net xylose equivalents (g/L)
Control:				
D-xylose	0.868	0.500	0.873	0.503
Furfural(10mg/L)	0.606	0.350	0.621	0.358
Hexoses:				
D-glucose	0.008	0.007	0.025	0.017
D-galactose	0.016	0.014	0.025	0.017
D-mannose	0.003	0.004	0.011	0.009
D-cellobiose	0.001	0.002	0.042	0.027
Methyl pentoses:				
D-rhamnose	-0.004	0.000	0.025	0.017
D-fucose	-0.004	0.000	-0.029	-0.014
Sugar derivatives:				
Hydroxymethyl-furfural	0.010	0.008	0.041	0.026
Methylfurfural	-0.004	0.000	-0.027	-0.013
Non-carbohydrates:				
D-glucuronic acid	0.027	0.018	0.037	0.024
D-galactoronic acid	-0.004	0.000	0.013	0.010
Acetate	-0.004	0.000	0.039	0.025

a - Assay on each component in the absence of D-xylose supplement:
Absorbance (540nm) = Avg. absorbance of test - avg. of blanks
Xylose equivalents (g/L) = (Absorbance - 0.0044)/1.7451

b - Assay on each component in the presence of 0.5 g/L D-xylose:
Net Absorbance (540nm) = (Avg. absorbance of sample - avg. absorbance of D-xylose)
(e.g. sample absorbance - 0.868)
Net xylose equivalents (g/L) = (Net absorbance - 0.0044)/1.7451

Table 3. Chemical Composition of the Spent Liquors from Econotech Cook No.10, 11, 12, and Cook No.13, 14, 15, 16.

	No.10,11,12	No.13,14,15,16
pH	2.7	2.3
Solid content %(w/v)	20.1	30.2
Furfural %(w/v)	0.01	0.01
Methanol %(w/v)	0.07	0.07
Acetic acid %(w/v)	0.71	0.72
Pentose %(w/v)	0.09	0.27
Pentosan %(w/v)	26.8	28.2
Glucose %(w/v)	0.0	0.0
Total lignin content %(w/v)	9.35	10.22
Klason lignin %(w/v)	2.16	2.46
Acid soluble lignin %(w/v)	7.19	7.76

Both pooled samples were in very dilute form and therefore necessitated further concentration to a syrup of around 20-30% solids content prior to chemical analysis.

Table 4. Butanediol Fermentation on Xylose Supplemented with Spent Liquors from Econotech Cook No.10,11,12 and Cook No.13,14,15,16.

Sample	Sample Supplement	Relative Butanediol Yield (%)
Control	None	100
Cook No.10,11,12	1% sample (v/v)	18
	2% sample (v/v)	13
Cook No.13,14,15,16	1% sample (v/v)	9
	2% sample (v/v)	9

Table 5. Summary of Technical Data Provided by Econotech Services Limited on the Organosolv Pulping Process

Cook No.	Cook pH	Ratio: ppt. water to liquor (v/v)	Cook time (h)	Composition of spent liquor after lignin precipitation & concentration to 1L. (g/L)					
				Arab-inan	Xylan	Galac-tan	Glucan	Mannan	Total lignin
10	N.A.	2.5	2	1.3	35.6	1.4	1.8	1.6	N.A.
11			1						
12			0.5						
13	N.A.	1.0	4	1.3	40.7	1.9	1.4	1.5	N.A.
14			3						
15			6						
16			2						
17	4.2	2.0	2	13.1	10.3	1.1	0.8	1.1	63.3
18	3.9	2.0	3	5.0	31.3	1.3	1.4	1.5	71.3
19	3.8	2.0	4	17.4	38.1	10.0	5.0	2.1	70.0
20	3.2	2.0	1	14.5	50.6	4.9	2.8	1.9	86.3
21	3.7	1.5	2	8.1	112.0	5.1	12.6	4.7	N.A.
22	3.1	1.5	1	8.0	135.0	6.1	26.7	5.8	N.A.
23	3.1	1.5	1	6.9	122.0	5.1	23.9	5.3	N.A.
24	3.2	1.5	3	5.2	84.3	3.7	43.5	4.5	116.7
25	3.1	1.5	2.7	4.5	77.6	4.1	45.3	4.1	135.0
26	3.0	1.5	1	5.6	99.7	5.7	29.2	4.7	135.0

Aspen wood chips were cooked with methanol-water, 80:20 (v/v), at 190°C for different time durations in the presence of the catalyst $MgSO_4$ (0.024 M for cooks No.10, 11, 12, and 0.05 M for cooks No.13, 14, 15, 16) or 0.01 M H_2SO_4 (at various final pH for cooks No.17 to 26).

N.A. - Data not available.

Table 6. Chemical Characterization of the Spent Liquors from Econotech Cook No.17, 18, 19, 20, 21, and 22.

	Econotech Cook No.					
	17	18	19	20	21	22
pH	1.9	1.6	1.6	1.2	1.8	1.8
Solid content %(w/v)	37.1	45.5	44.3	62.0	51.4	67.7
Furfural %(w/v)	0.01	0.02	0.01	0.01	0.01	0.02
Methanol %(w/v)	0.73	0.99	0.36	0.59	0.55	0.55
Acetic acid %(w/v)	2.60	1.85	0.38	0.52	0.77	0.48
Pentose %(w/v)	2.5	5.7	6.5	9.1	7.7	10.2
Pentosan %(w/v)	41.8	39.8	35.2	39.2	41.2	36.7
Total lignin content %(w/v)	9.13	9.17	9.76	8.84	9.63	7.60
Klason lignin %(w/v)	2.55	2.78	3.17	2.16	2.97	1.89
Acid soluble lignin %(w/v)	6.58	6.39	6.59	6.68	6.66	5.66

Table 7. Butanediol Fermentation on Xylose Supplemented with Spent Liquors from Econotech Cook No. 17, 18, 19, 20, 21, and 22.

Sample No.	Sample Supplement	Relative Butanediol Yield (%)
Control	None	100
17	2% sample (v/v)	10
	5% sample (v/v)	14
18	2% sample (v/v)	14
	5% sample (v/v)	12
19	2% sample pentose (w/v)	16
	5% sample pentose (w/v)	11
20	2% sample pentose (w/v)	18
	5% sample pentose (w/v)	17
21	2% sample pentose (w/v)	11
	5% sample pentose (w/v)	15
22	2% sample pentose (w/v)	11
	5% sample pentose (w/v)	10

Table 8. Chemical Characterization of the Spent Liquors from Econotech Cook No.23, 24, 25, 26, 29, 30, and 31.

	Econotech Cook No.						
	23	24	25	26	29	30	31
pH	1.9	0.9	3.3	3.2	1.9	1.8	1.9
Solid content %(w/v)	54.1	67.6	69.2	71.4	60.5	52.5	47.7
Furfural %(w/v)	0.02	0.02	0.02	0.02	0.02	0.01	0.01
Methanol %(w/v)	0.32	0.69	0.23	0.22	0.21	0.34	0.20
Acetic acid %(w/v)	1.15	1.19	0.83	0.62	0.54	0.29	0.50
Pentose %(w/v)	7.8	8.5	5.9	6.7	1.1	5.2	6.3
Pentosan %(w/v)	37.6	26.5	25.1	37.9	2.0	----	----
Total lignin %(w/v)	9.10	7.89	9.22	8.32	9.39	9.76	9.89
Klason lignin %(w/v)	2.66	2.68	3.24	4.00	2.84	3.42	3.07
Acid soluble %(w/v)	6.44	5.21	5.98	4.32	6.55	6.34	6.82

Table 9. Butanediol Fermentation on Xylose Supplemented With Spent Liquors from Econotech Cook No.23, 24, 25, 26, 30, and 31.

Sample	Sample Supplement	Relative Butanediol Yield (%)
Control	None	100.0
No.23	2% sample pentose (w/v)	9.0
No.24	2% sample pentose (w/v)	6.0
No.25	2% sample pentose (w/v)	6.0
No.26	2% sample pentose (w/v)	5.0
No.30	2% sample pentose (w/v)	1.6
No.31	2% sample pentose (w/v)	3.2

Table 10. Chromatographic Data and Pentose Concentration in the Fractions Obtained Via Amberlite IR-45 Column Chromatography of the Spent Liqours from Econotech Cook No.17, 18, 19, 20, 21, and 22.

Cook No.	Pentose conc. (mg/ml)	Ratio: resin to sample solids (w/w)	Total pentose loaded (mg)	Fractions collected				Pooled[a] F1&F2 pentose conc. (mg/ml)
				No.	Vol. (ml)	Pentose conc. (mg/ml)	Pentose recovered (mg)	
17	25.0	2.7	1000.0	F1	41	12.76	1002.7	21.5
				F2	42	10.41		
				F3	40	1.06		
18	56.9	2.2	2276.0	F1	43	29.26	2307.5	44.8
				F2	53	18.74		
				F3	30	1.87		
19	64.8	1.6	5378.4	F1	81	41.58	6105.7	48.2
				F2	82	32.59		
				F3	76	0.86		
20	90.6	1.2	7610.4	F1	80	61.73	8937.1	83.8
				F2	80	48.00		
				F3	76	2.12		
21	76.8	1.4	6374.4	F1	79	31.31	7476.5	61.1
				F2	79	61.83		
				F3	84	1.41		
22	102.3	1.1	8695.5	F1	80	60.25	8929.6	81.1
				F2	80	48.99		
				F3	84	2.41		

a. The pooled fractions of F1 and F2 for each sample were very dilute and therefore necessitated further concentration using rotary evaporation at 50°C under high vacuum.

Table 11. Chromatographic Data and Pentose Recovery in Fractions Obtained Via Mixed Bed Ion-exchange Column Chromatography of the Spent Liquors from Econotech Cook No.17,18,19,20, and 21.

Cook No.	Type of Resin	Ratio(w/w) Resin-to-Sample Solids	Total Pentose Loaded (mg)	Total Pentose Recovered (mg)	Total Pentose Lost (%)	Pentose[a] in Pooled Fractions (mg/ml)
17	MB-1	6.4	762.5	397.4	47.9	5.52
	AG-501-X8	6.4	762.5	13.5	98.2	----[b]
18	MB-1	7.0	1653.0	476.9	71.2	9.98
	AG-501-X8	7.0	1653.0	223.4	86.5	5.30
19	MB-1	7.6	5184.0	100.5	98.1	----[b]
20	MB-1	7.1	1812.0	50.0	97.2	----[b]
		4.0	1812.0	932.0	48.6	77.44
21	AG-501-X8	4.0	1920.0	1056.0	45.0	63.75

a. Pooled fractions were rotary evaporated at 50°C under high vacuum.

b. Pentose concentrations in these pooled fractions were too low to carry out fermentation, even though they were rotary evaporated.

Table 12. Butanediol Fermentation on 2% Pentose in the IR-120 and IR-45 Treated and Overlimed Spent Liquors from Econotech Cook No.30 and 31.

Sample No.	Inoculum Source 5%(v/v)	Treatment Methods	Relative Butanediol Yield (%)
Control	Growing cells	None	100.0
	Resting cells	None	100.0
30	Growing cells	IR-120, IR-45	7.4
		IR-120, IR-45 & overlimed	7.4
	Resting cells	IR-120, IR-45	13.0
31	Growing cells	IR-120, IR-45	3.7
		IR-120, IR-45 & overlimed	3.7
	Resting cells	IR-120, IR-45	14.8

Table 13. Butanediol Fermentation from the Pooled Fractions of the Sephadex LH-20 Eluted Sample No.23.

Sample No.	Pooled[a] Fraction No.	Xylose Conc.	Pooled Fraction Supplemented	Relative Butanediol Produced(%)
Control	None	2%	None	100.0
	None	1%	None	100.0
23	F5	None	1%(w/v)pentose	55.2
	F6	None	1%(w/v)pentose	35.2
	FD1	2%	2.5%(v/v)	57.4
		2%	6.5%(v/v)	70.7
	FD2	2%	2.5%(v/v)	51.4
		2%	7.5%(v/v)	44.8
	FD3	2%	2.5%(v/v)	47.2
		2%	7.5%(v/v)	58.5

a. All pooled fractions were freeze-dried and reconstituted to the same volume as the sugar fractions in FD2, except for F5 and F6 which were fermentated without any concentration step.

Table 14. HPLC Analysis of Fractions from the Spent Liquor from Cook No.23 After Elution Through Sephadex LH-20.

Sugars standard at 1 mg/ml	Retention time (min) and Area count (%)[a]					
	Untreated sample	F4	F5	F6	F7	F8
Xylobiose (11.40)	11.37 8.3%	11.37 23.1%	11.38 11.6%	11.38 6.9%	11.38 5.4%	11.38 5.9%
Glucose (12.38)	12.38 5.9%	12.36 15.1%	12.38 6.9%	12.39 4.5%	12.39 3.3%	12.35 2.1%
Unknown (13.02)	13.02 11.4%	13.01 25.0%	13.02 15.0%	13.03 10.9%	-----	-----
Xylose (13.42)	13.39 19.6%	13.34 14.5%	13.39 19.5%	13.40 18.8%	13.40 25.2%	13.40 21.7%
Unknown (13.88)	13.86 21.1%	13.86 12.1%	13.87 20.2%	13.88 23.9%	13.88 25.5%	13.88 24.9%
Galactose (14.23)	-----	-----	-----	-----	-----	-----
Arabinose (15.44)	15.45 1.3%	-----	15.46 0.7%	-----	15.39 0.1%	-----
Mannose (15.89)	-----	-----	-----	-----	-----	-----
Unknown (16.45)	16.44 32.2%	16.47 10.1%	16.47 26.2%	16.47 35.0%	16.47 40.3%	16.48 45.4%

The HPLC was operating at a flow rate of 0.6 ml per minute with water as the eluent on an Aminex HPX-87P column.

a. The area counts of each compound peak was expressed as the percent of that peak to the total peak areas in that fraction.

Table 15. Butanediol Fermentation of the Pooled Fractions from the Sephadex LH-20 Eluted Spent Liquor Sample from Cook No.24.

Pooled fractions No.	Xylose conc. (% w/v)	Pooled fractions supplemented	Relative butanediol yield (%) day2	day4
Control	2	None	100	100
FD1	2	10% (v/v)	80	90
	2	40% (v/v)	101	99
FD2	None	40% (v/v) i.e. 2% pentose	72	102
FD3	2	10% (v/v)	60	70
	2	40% (v/v)	34	42
FD4	2	all[a]	80	84

a. Since the ethanol extracted fractions were rotary evaporated to dryness and were unable to dissolve properly in the culture medium, a trace amount of ethanol was added to the extract to solubilize the extract and finally the medium was flushed under nitrogen to remove the trace amount of ethanol. From the G.C. analysis of the culture broth for solvent production, there was only small quantities of ethanol present which was comparable to that obtained in the control.

Figure 1. Effect of Amberlite IR-45 Ion-Exchange Treatment on Fermentability of Organosolv Spent Liquors

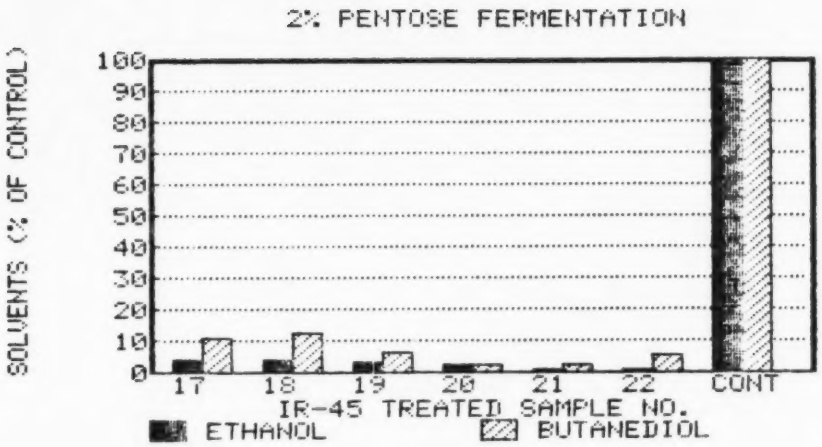


Figure 2. Effect of Mixed Bed Ion-Exchange Treatment on Fermentability of Organosolv Spent Liquors

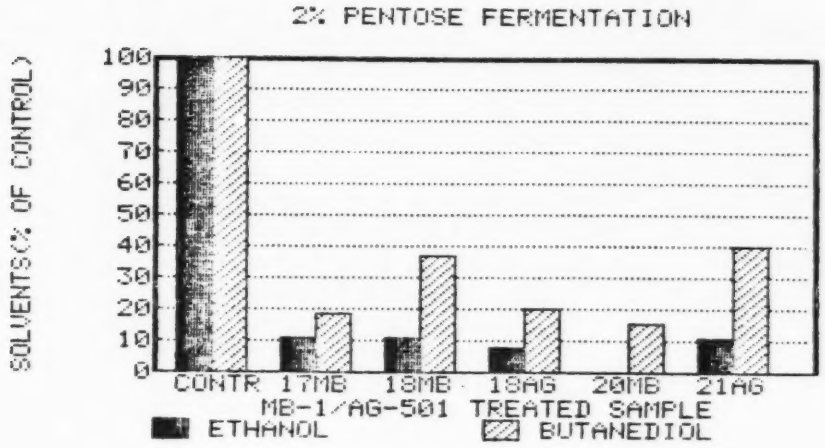


Figure 3. **Effect of Combined Ion-exchange and Overliming Treatment on Fermentability of Organosolv Spent Liquors - First Trial**

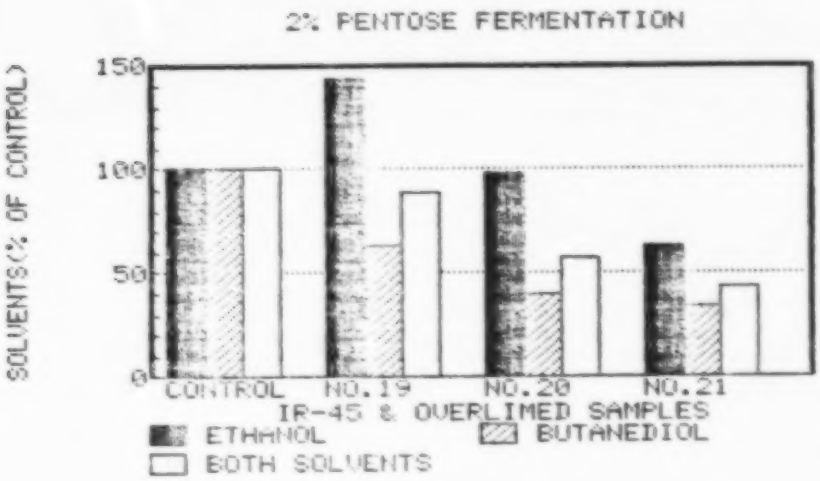


Figure 4. Effect of Combined Ion-exchange and Overliming Treatments on Fermentability of Organosolv Spent Liquors - Second Trial

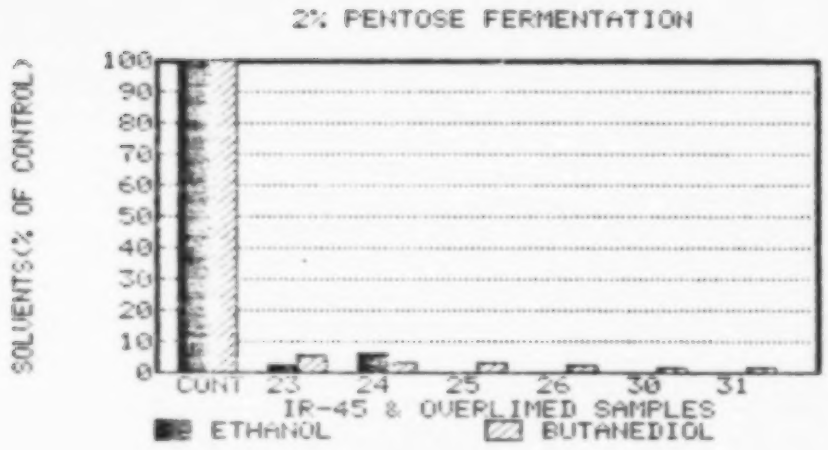


Figure 5. LH-20 Elution Profile for Organosolv Spent Liquor from Cook No. 23

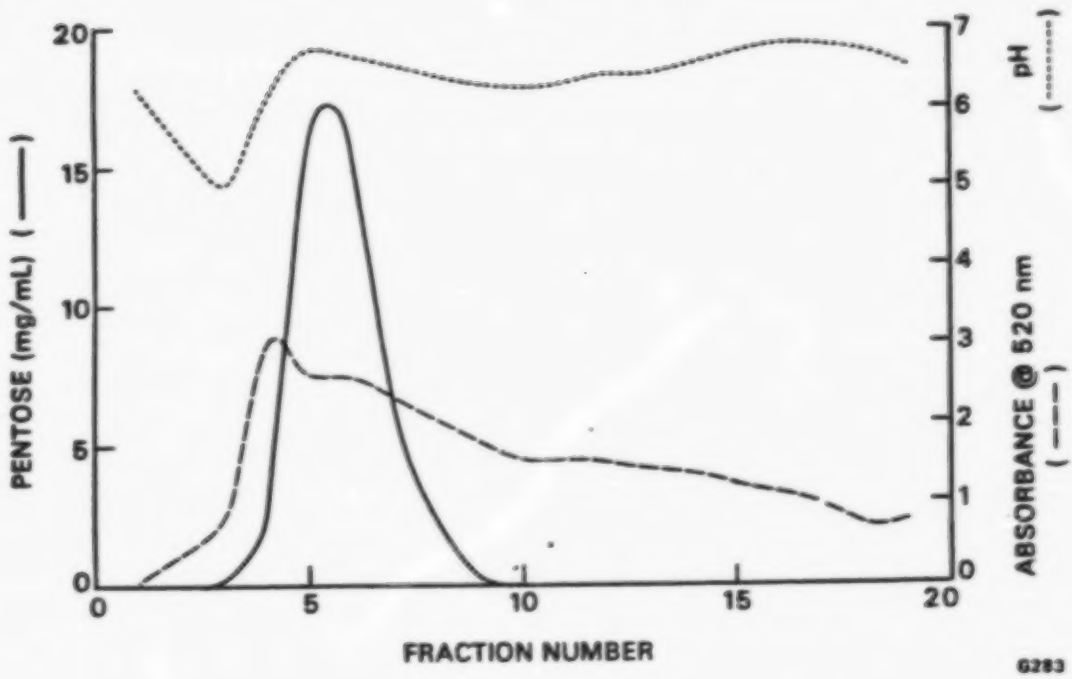


Figure 6. LH-20 Elution Profile for Organosolv Spent Liquor from Cook No. 24

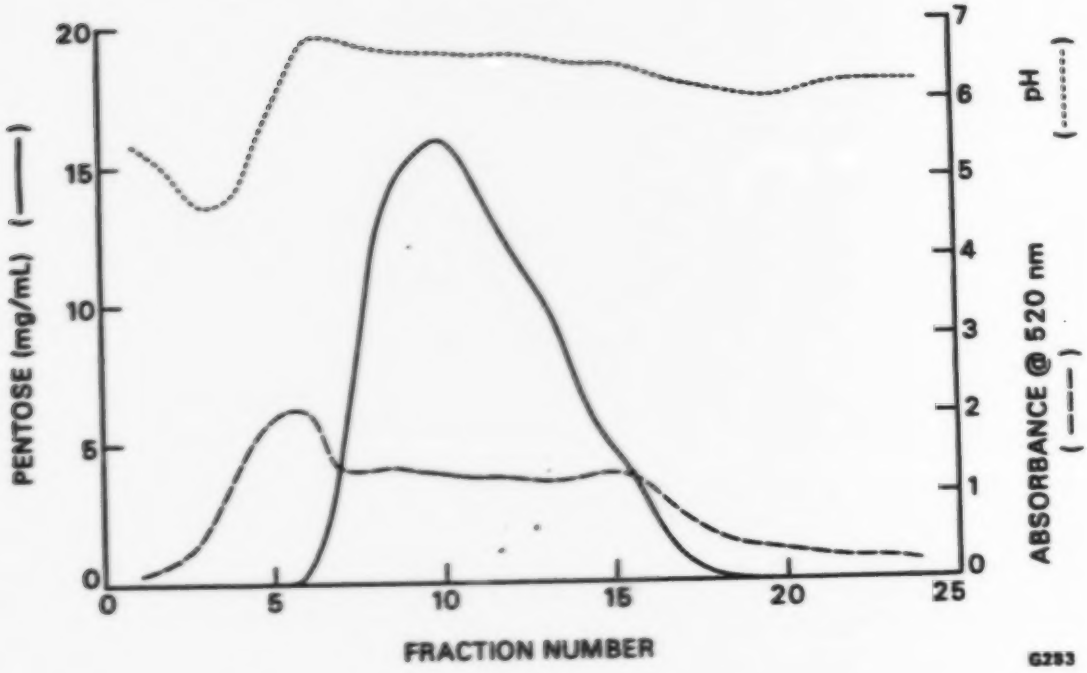
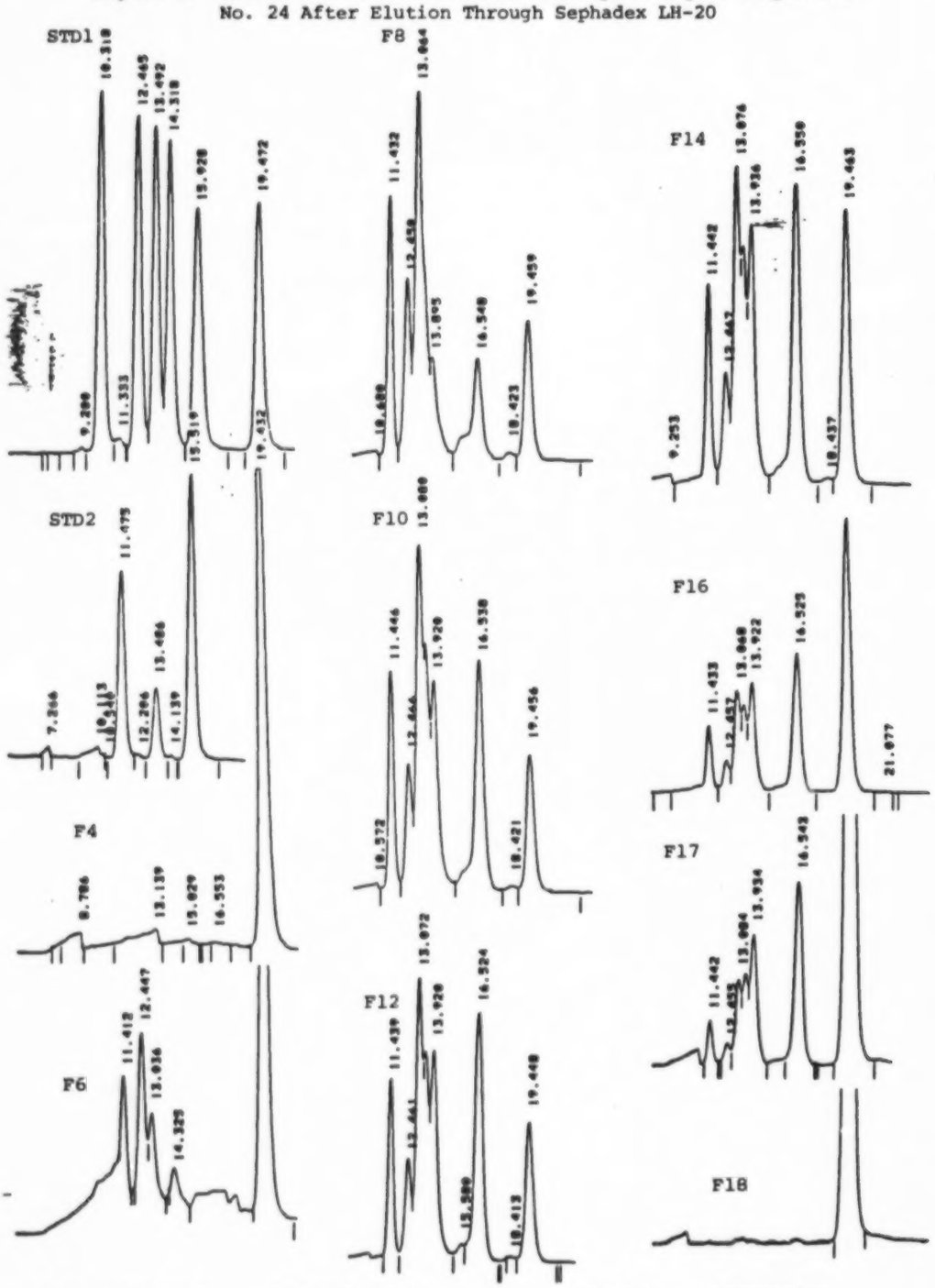


Figure 7. HPLC Profiles of Fractions from Spent Liquor Sample Cook No. 24 After Elution Through Sephadex LH-20



Sugar standards	Retention time
Cellobiose	10.32
Xylobiose	11.47
Glucose	12.46
Xylose	13.49
Galactose	14.31
Arabinose	15.52
Mannose	15.93
Erythritol	19.45
Unknown 1	13.04
Unknown 2	13.90
Unknown 3	16.55

Fractions	Attenuation
F4	2
F6	2
F8	8
F10	8
F12	8
F14	4
F16	4
F17	1
F18	1